Review

Establishment of the milk-borne transmission as a key factor for the peculiar endemicity of human T-lymphotropic virus type 1 (HTLV-1): the ATL Prevention Program Nagasaki

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(Communicated by Takashi SUGIMURA, M.J.A.)

Abstract: In late 2010, the nation-wide screening of pregnant women for human T-lymphotropic virus type 1 (HTLV-1) infection was implemented in Japan to prevent milk-borne transmission of HTLV-1. In the late 1970s, recognition of the adult T-cell leukemia (ATL) cluster in Kyushu, Japan, led to the discovery of the first human retrovirus, HTLV-1. In 1980, we started to investigate mother-to-child transmission (MTCT) for explaining the peculiar endemicity of HTLV-1. Retrospective and prospective epidemiological data revealed the MTCT rate at ∼20%. Cell-mediated transmission of HTLV-1 without prenatal infection suggested a possibility of milk-borne transmission. Common marmosets were successfully infected by oral inoculation of HTLV-1 harboring cells. A prefecture-wide intervention study to refrain from breast-feeding by carrier mothers, the ATL Prevention Program Nagasaki, was commenced in July 1987. It revealed a marked reduction of HTLV-1 MTCT by complete bottle-feeding from 20.3% to 2.5%, and a significantly higher risk of short-term breast-feeding (<6 months) than bottle-feeding (7.4% vs. 2.5%, $P < 0.001$).

Keywords: breast milk, HTLV-1, infectious disease transmission-vertical, adult T-cell leukemia, infection/prevention and control

Discovery of adult T-cell leukemia (ATL)

ATL was discovered by peculiar "flower cells" in 1977.[1)] ATL is a very rare disease in Kyoto area, where Prof. K. Takatsuki found that most ATL patients were originated in Kyushu. Hematologists in Kyushu had not recognized ATL as a distinct disease entity, since ATL was popular in Kyushu. In actuality, I found later that the total incidence of leukemia/lymphoma in Nagasaki Prefecture in 1980s was twice as much as that in the entire Japan, and approximately one half of them were cases of ATL. However, according to statistical data supplied by National Cancer Center for 2009 does not give such a distinct difference between Nagasaki Prefecture and whole Japan. Since combined death rates of malignant lymphoma and leukemia were 5.6 and 4.7 per 10^5, respectively, the excess deaths due to these diseases in the Nagasaki Prefecture for the year 2009 can be calculated as only 13,[2)] which is far less than 100 diagnosed ATL cases per year in the Prefecture.[3)] Because of the endemic nature of ATL, Prof. K. Takatsuki assembled a group of collaborators to search a causative agent. Prof. Y. Hinuma, an expert in the field of Epstein Barr virus (EBV), found an antibody detected in all ATL patients and with high frequency in their family members by an indirect immunofluorescence test.[4)] The target cell in the test, MT-1, had been established by Prof. I. Miyoshi[5)] without knowledge of the distinct entity of ATL. Type C particles were found in MT-1 cells after treatment with iododeoxyuridine (IUdR).[4)] Later, the virus was identified as the first human infectious retrovirus; it had a novel distinct gene, *pX*.[6)] The virus was named as "ATL virus (ATLV)" by this group. However, the current terminology, human

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doi: 10.2183/pjab.87.152

T-lymphotropic virus type 1 (HTLV-1), was used throughout this article after nomenclature by International Committee on Taxonomy of Virus.[7]

The first human retrovirus

Retroviruses have been known as oncogenic since the discovery of Rous sarcoma virus in the early 1900s. A wide variety of animal retroviruses has been described up to the present. Searches for a responsible retrovirus in human cancers were attempted extensively. The "oncogene theory" by Huebner and Todaro in 1969[8] accelerated the competition even further. Hundreds of published and unpublished human retroviruses have been 'isolated' from "human sources", but these inferred "novel human viruses" were identified as laboratory contaminations without any exception. The hope for finding a human retrovirus was almost dying at the end of 1970s. Therefore, the discovery of a new retrovirus from a culture of Sezary syndrome in 1980[9] did not attract enough attentions by itself. Although Prof. Y. Hinuma's report published in 1981[1] did not carry enough information to establish the discovery of a novel human retrovirus, it contained an important evidence: the association of a specific type of human leukemia, ATL, with an antibody against MT-1 cells which harbored an inducible C-type virus. The discovery of another human retrovirus, human immunodeficiency virus (HIV), followed in 1983. Surprisingly, HIV contained a gene similar to the *pX* of HTLV-1, even though these two viruses are taxonomically classified into different genera, *deltaretrovirus* and *lentivirus*, respectively.[7]

Group meeting of HTLV-1 at Kyoto University in the fall of 1980

I knew nothing about ATL when I joined the laboratory of Prof. T. Miyamoto at Nagasaki University in the early 1980. In the fall of 1980, Prof. Y. Hinuma invited Prof. T. Miyamoto to a group meeting at Kyoto University on the recently discovered human retrovirus, HTLV-1. Prof. T. Miyamoto had worked for Prof. Y. Hinuma at Tohoku University. He was asked to survey the transmission pathway of HTLV-1, because Nagasaki was located in the center of HTLV-1 endemy.[4),10]

Prof. T. Miyamoto sent me to the meeting. The information on the new human retrovirus presented at the meeting excited me promptly. The high frequency of lymphatic leukemia in Kyushu reminded me a comment of my hematologist father some 50 years ago that the bone-marrow involvement in leukemic patients in Kyushu was significantly lower than that in those from other regions of Japan. Virus carriers have antibodies against the virus detectable by the indirect immunofluorescence test, and therefore, carriers in general population were easily screened. Virus carriers were distinctly more prevalent in Kyushu, and they were clustered heavily in the family members of ATL patients, implying that the viral transmission was more frequent within family members. While the prevalence of HTLV-1 in the husbands of carrier wives was no higher than that in the general population, wives of carrier husbands were almost invariably infected among senile populations, suggesting a male-to-female sexual transmission.[10] However, because the prevalence in females was indistinct before forties, the efficiency of male-to-female transmission seems to be relatively inefficient.

The virus was transmitted with whole blood transfusion; as much as 60% of recipients were infected after one unit (200 mL) of transfusion, but not through fresh plasma infusion.[11] *In vitro* infection experiments revealed that the virus is easily transmissible by co-culture of infected cells with fresh lymphocytes in the presence of IL-2, but not by inoculation with infected culture fluid. These lines of evidence have suggested that infection of the virus is cell-associated, requiring the direct contact of donor's infected living cells with uninfected recipient's cells.

On the way back to Nagasaki from the meeting

Excited with a prospect that I have finally found my life-long project, "to determine the transmission pathway of HTLV-1", I went back to Nagasaki asking questions to myself. Why HTLV-1 was kept endemic in Kyushu, especially in rural areas? The endemic feature of most microorganisms became less clear in modern communities due to busy human and material traffics. Most pathogens still endemic are usually dependent on vectors, such as animals or insects. Most of these endemic diseases have been wiped out or became rare in the contemporary Japan. A pathogen could be localized in a certain region, if it were recently introduced into the community. But it would be more often in major cities or surrounding areas, not in rural areas. Therefore, HTLV-1 has probably been present historically in rural areas, and stayed there for a long period without free exportation. Because of a relative inefficiency in transmissions, the virus probably remained dormant, even if it had been exported into new areas.

Accumulation of HTLV-1 carriers within families of ATL patients suggested an intrafamilial transmission. If a microorganism is spread mainly by the sexual transmission, sooner or later it should be spread around the world as other sexually transmitted diseases. Inefficiency of sexual transmission in HTLV-1 was consistent with the endemicity. What is a more intimate relation than husband and wife? Because intrafamilial transmissions *via* aerosol, droplet or direct contact should be also common in other relationships, such as siblings and classmates, these common transmission pathways are unlikely as the leading candidate. If transmissions among siblings are common, they should be common also in nurseries and schools, diluting the influence of intrafamilial transmissions.

Retroviruses lose the infectivity more rapidly than other RNA viruses, such as influenza virus, probably because the viral reverse transcriptase indispensable for viral infection is unstable. Because HTLV-1 is strongly cell-associated, transmission of HTLV-1 needs the transfer of live infected cells. Keeping T-lymphocytes alive outside the body is trickier than maintaining virus particles alive, even in optimized experimental conditions. Therefore, transfer of live T-lymphocytes from donor to recipient should require the direct exchange of body-fluids. I came to a conclusion that the best candidate for transferring significant amount of body fluid should be in the intimate relation between mother and child. I went on to setup a field experiment to provide a hard evidence of MTCT as the major cause of HTLV-1 endemy.

Later, at one of conventions for Japanese Cancer Association, one of attendant doctors came to me, and insisted the possibility of mosquito-borne infection to explain the endemicity of HTLV-1. The endemic feature in Kyushu, representing the southwestern hot region, was consistent with this assumption. I had discarded the possibility by three reasons. Blood volume carried by mosquito would be too small for cell-mediated transmissions. Survival of human T-lymphocyes within the body of mosquito should be difficult. If HTLV-1 replicates in mosquito cells, the cell-to-cell infection from mosquito cells back to a new human recipient is necessary. Above all, mosquitos cannot explain the familial clustering of carriers.

Endogenous and infectious retroviruses

Animal retroviruses are classified into two categories, endogenous and infectious. Endogenous viruses reside as the proviral DNA covalently integrated in chromosomal DNA of every cell throughout the body, and in all individuals within the same species. Since most endogenous viruses are non-infectious and non-replicative in the original host, they do not induce any harm to the host. Most endogenous viruses are defective and present as non-expressing genes, while they occupy over 1% of mammalian genome, even in the human species. Infectious viruses spread in animals by transmissions between individual hosts. The retrovirus is usually specific to cells within a certain branch of cell differentiations, such as $CD4^+$ T-cells in case of HTLV-1. Unlike most other viruses, the retrovirus remains in the body lifelong once the infection is established, even after the viral replication is ceased by immune responses of the host. The virus is present only in infected cells within the infected individual, mostly in the form of proviral DNA.

Vertical and horizontal transmission of microorganism

Vertical transmission is the transmission from a parent to child. Vertical transmission in a narrow sense is the transfer of a microorganism from parent to child before delivery. Because transmission associated with sperm is rare, except for endogenous retroviruses, the vertical transmission nearly equals to MTCT. Horizontal transmission is a mode of transmission between individuals, *i.e.*, after the birth. However, the vertical transmission in a broad sense includes the transmission soon after birth, because discrimination is not always possible. Herpes viruses can be transmitted from mother to fetus *in utero*, at the time of delivery, or postnatally. If a child were sick at the time of delivery, it is easy to postulate *in utero* transmission. Transmission at the time of delivery may induce serious disease shortly after the delivery, typically within a week or two. If the sign of infection became evident at 6 months after delivery, it is not easy to specify the time of transmission depending on the virus concerned. As a result, all these maternal transmissions could be categorized as vertical in a broad sense. Most body-fluids transferred postnatally from mother to child, such as blood and saliva, are not specific for mother-to-child or intrafamilial transmissions. Therefore, milk-borne transmission is the most likely candidate in the case of MTCT for HTLV-1; however, there were a number of uncertainties when we started the study. Is the MTCT concept plausible? Are infected T-cells present in breast milk of carrier mothers in

enough amounts to explain MTCT? Can T-cells survive through acidic conditions of the stomach? Where is the gate of homing for the infected T-cells, oropharyngeal lymphatic tissues, Peyer's patches in the intestine or else?

Most non-specialized researchers think that the vertical transmission nearly translates to an intrauterine transmission. However, intrauterine transmission is rather rare in the vertical transmission of viruses. Vertical transmission of viruses in mammalian species could be divided into four categories: genetic, intrauterine, during delivery and postnatal. Genetic transmission is the mode for endogenous retroviruses present as proviral DNA genomes within ovum and sperm. This is not applicable to HTLV-1. Some viruses, such as rubella virus, human cytomegalovirus (HCMV) and parvovirus B19, are known to transmit through the placenta. Because trophoblasts in placenta are the target cells of these viruses, they tend to migrate through the placental barrier more efficiently. In these cases, the evidence of intrauterine transmission may be manifest *in utero*, at the time of delivery or shortly after delivery, sometimes causing serious disease. Even in these viruses, postnatal transmissions are far more frequent than intrauterine transmissions; otherwise the survival of human species would have been difficult.

For viruses circulating in the peripheral blood, minor injuries during delivery process can cause vertical transmissions. Because the viral titer of hepatitis B virus (HBV) can be as high as 10^{12} particles/mL, most babies delivered transvaginally will be infected during delivery. Even elective cesarean section is not helpful to shut off the transmission. As the virus titer is usually $<10^7$ particles/mL for hepatitis C virus (HCV), only up to 10% of babies born to HCV RNA-positive mothers will be infected. Most of the HCV transmission might be avoided by elective cesarean sections, but the natural history of HCV MTCT has not been established seriously enough to justify the procedure.[12)]

In animals, several infectious retroviruses had been known, such as avian leucosis virus (ALV), mouse mammary tumor virus (MMTV), feline leukemia virus (FeLV), gibbon ape leukemia virus (GaLV), Mason Pfizer monkey virus (MPMV) and bovine leukemia virus (BLV). HTLV and HIV have been added to the list in the early 1980s. Infectious or non-endogenous ALVs in flocks may behave similarly to endogenous viruses, because the virus is already replicating in a wide variety of embryonic cells in the egg. MMTV is the first tumor virus whose main transmission pathway *via* breast milk was established.[13)] Essentially every female C3H/He mouse will suffer from breast cancer. Milk-borne transmission of MMTV was proved by prophylactic foster-nursing of female offsprings to the other strain of mice free from the MMTV. However, virions are considered responsible for transmission, rather than cells floating in the breast milk. FeLV is prevalent in some households that rear a large number of cats, sometimes up to 30%. The presence of high-titered virus in saliva and fighting between kittens were suspected as the source of infections within the households. Transmission of FeLV through breast milk might be possible, but I am not aware of a report describing the breast milk transmission. Transmission routes of simian viruses have not been studied thoroughly, as far as I know.

Screening of pregnant women for HTLV-1 in Nagasaki

On December 01, 1980, just thirty years ago, we started a pilot study to screen pregnant women for anti-HTLV-1 antibody in the Nagasaki City area. The purpose of screening was to establish a hard evidence that mother-to-child transmission (MTCT) is the major transmission pathway for survival of HTLV-1 in endemic areas of Japan. A variety of current technologies, such as polymerase chain reaction (PCR) and commercial anti-HTLV-1 antibody detection kits, were yet to be available in those days. An 8-bit personal computer on BASIC accepted only numbers and alphabets, but not Chinese characters to register the name and address of pregnant women enrolled. A cassette tape recorder was the only memory device.

Prevalence of HTLV-1 carriers in Nagasaki

We started out to survey the background prevalence of HTLV-1 carriers in the Nagasaki area, especially among young populations.[14)] The prevalence in medical students at Nagasaki University was just above 1%, and that in the nurse school was below 1% (Table 1). In order to find the prevalence in children, residual blood samples of pediatric patients at the biochemistry laboratory of Nagasaki University Hospital were tested. As high as 5% were found positive, but due to artifacts. At first, we obtained corded samples to keep confidentiality. Soon, we found that several consecutive samples were lined positive. This was due to multiple sample tubes originated in the same patients, and most of these patients were leukemic who had received multiple

Table 1. Prevalence of HTLV-1 carriers of Nagasaki area in the early 1980s[a)]

	Age (y)	HTLV-1 carriers			P
		No. positives	No. tested	Prevalence (%)	
Case:					
Children of carrier mothers	1–10	17	78	21.8	—
Controls:					
Pediatric patients[b)]	0–19	14	533	2.6	<0.001
Nurse school students	18–19	1	192	0.5	<0.001
Medical school students	20–	7	488	1.4	<0.001
Blood donors	16–18	21	1,274	1.6	<0.001
Pregnant women		187	5,015	3.7	<0.001

a) Anti-HTLV-1 antibody was screened by the particle agglutination assay of Fuji Rebio and confirmed by the indirect immunofluorescence test.
b) Among 14 pediatric patients who were positive for the anti-HTLV-1 antibody, 12 had received multiple transfusions for leukemia.

Table 2. Prevalence of HTLV-1 carriers among mothers of carriers

	No. positives	No. tested	Prevalence (%)
Mothers of pregnant carriers[a)]	6[b)]	8	75
Mothers of carrier school children[c)]	12[d)]	13	92

a) Some biological mothers of pregnant carriers voluntary wanted to know their carrier status. We did not actively recommend the testing, because the information of carrier status should be kept confidential even in family members.
b) Both of two pregnant women whose mothers were negative for anti-HTLV-1 had received multi-units of transfusions, one for open heart surgery and others for massive bleeding during the previous delivery.
c) Fifty-six children were found positive for the anti-HTLV-1 antibody among approximately 1,600 children in primary, junior and high schools of a remote town in the Nagasaki Prefecture. Among females in the same town who received annual health check-ups for adults, 13 were identified as their mothers in the town registry by the name and birth date.
d) Details unknown for the remaining mother who was negative for the antibody.

blood transfusions. Actually, over 80% of leukemic patients were found antibody-positive. Furthermore, repetitive uses of pipets in the biochemistry laboratory produced contaminations to consecutive samples. Singlet blood samples obtained from serology laboratory without no repetitive uses of pipets revealed a more realistic prevalence, <1%, when leukemic patients were excluded.

Retrospective study of possible MTCT

My plan was to start the screening of pregnant women for anti-HTLV-1 antibody in the Nagasaki area. At the beginning, I presented my plan to Prof. T. Miyamoto of our department, Prof. T. Yamabe of Department of Obstetrics and Prof. Y. Tsuji of Department of Pediatrics. They agreed to organize the study. Prof. T. Yamabe introduced me to several obstetric clinics and hospitals in the area. Obtaining an informed consent from each individual was not strict in those days. However, the purpose of the study was explained to each pregnant woman by attending obstetricians, because HTLV-1 was a putative leukemia virus. Ethic committee was not available in the university when we started the study. Later a handout documenting the purpose of screening and intervention was prepared by Nagasaki Prefecture.

Pregnant women were asked to enroll in the screening for anti-HTLV-1 antibody. The prevalence of HTLV-1 carriers in pregnant women of the Nagasaki City area was approximately 4%. Carrier mothers were asked to bring their elder children to test if some of them had been infected. Within a few years, we found that the prevalence in children born to carrier mothers was approximately 20% (Table 1). The prevalence in children of the Nagasaki area, 1%, was roughly consistent with the product of maternal prevalence at 4% and transmission rate of 20%. The results suggest that carrier mothers can transmit HTLV-1 to their offsprings, and that a significant portion of carrier children had received the virus from their mothers.[14)]

A concept will become more solid if the reverse proposition, "mothers of children carrier are carriers", bears out true. Among the group of pregnant women we screened, some mothers of pregnant carriers (grandmothers of expected babies) volunteered to be tested their infection status. We did not recommend testing grandmothers, because we did not want to introduce additional concerns within the family. Six of eight grandmothers were found to be carriers.[14)] Both of the remaining two mothers had been transfused, one for open heart surgery and the other for massive bleeding at the time of previous delivery (Table 2). The results were consistent to our hypothesis. I felt relieved to know that some mothers were intelligent enough to extend the concept of vertical transmission, and that the quality of educations given by attending obstetricians was appropriately high.

The First Department of Internal Medicine allowed us to use blood samples of school children and of adults which had been collected for HBV research coupled with annual health care program in 1960s. The town office allowed us to go through the names and date of birth of each family member. Among 56 carriers in school children, we found 13 mothers matched with the children carriers; 12 of them (92%) were carriers (Table 2). Because the reverse proposition turned out to be also true, we were pretty confident in our working hypothesis that MTCT was the major transmission route for HTLV-1.[14)]

Postnatal transmission more likely

To confirm the MTCT, carrier mothers were asked to bring their elder siblings of the target baby. Consistent with the working hypothesis, the prevalence of HTLV-1 carriers among these children was 17/78 (21.8%), significantly higher than that of hospitalized children and young adults in the area (Table 1). Some of these children were followed. Most negative children remained seronegative, whilst small number of them seroconverted at 12 months of age or later. The transmission incidence, at approximately 20%, remained unchanged after 12 months of age.[15)] In the case of intrauterine infections, such as human cytomegalovirus and rubella virus, cord-blood samples of infected babies are usually positive both for virus and antibody, specifically IgM-antibody that cannot cross the placental barrier. In case of HBV and HCV, in which MTCT mainly takes place at the time of delivery, high titers of these virus and corresponding antibodies can be detected within 3 months after delivery. These results lend credence to the postnatal transmission to the prenatal transmission for HTLV-1. Late seroconversion has also been reported by other group.[16)]

Possibility of intrauterine infection in HTLV-1

The possible intrauterine infection was tested in cord-blood samples of babies born to carrier mothers. Because all the carrier mothers were positive for IgG-antibodies which can freely cross the placental barrier, all these babies are antibody-positive at the time of delivery. None of over 100 cord-blood samples were positive for IgM-antibodies by the indirect immunofluorescence test.[14)] However, we could not be certain because of a limited sensitivity of the assay system. The PCR technology was not available at that time. Infected cells in blood could be detected by culturing mononuclear cells in the presence of IL-2. We could not find a case of positive culture in 200 cord-blood cultures from babies born to carrier mothers, in the condition that approximately two thirds of cultures of peripheral blood mononuclear cells from carrier pregnant women produced positive. However, we could not exclude the possibility of intrauterine transmissions.

Because the maternal prevalence was approximately 4%, screening of at least 5,000 pregnant women were required to collect 200 cord-blood samples of babies born to carrier mothers. The existence of intrauterine infection of HTLV-1 was controversial in those days, because there were a few reports describing positive cord cultures from other laboratories.[17),18)] Since negative studies were more difficult to convince than positive ones, many researchers still believed the intrauterine transmission as a second transmission pathway of HTLV-1 to breast-feeding. We decided to look for postnatal transmission pathways.[14),15)]

The major pathway of MTCT

Direct transfer of the body fluid is probably required for MTCT of HTLV-1. Body fluids include blood, salivary juice, breast milk, seminal fluid, urine, stool and sweat. Contamination with the maternal blood during delivery process is a possibility. However, in the case of infection during delivery, earlier appearance of virus or antibody is expected. Blood transfusion after the birth should be rare during the normal life even with relationship of mother and child. Salivary juice can be a candidate. However, if salivary juice is infectious, the virus will spread among other family members, as well as among children in nurseries. Urine, stool and sweat were unlikely, because live cells in these fluids should be limited in number, and transfer of a significant amount of them is hardly conceivable in ordinary life styles. Seminal-fluid is out of question for MTCT.

Possibility of breast milk as a source of infection

The remaining and most likely candidate was breast milk. Although spread of retrovirus *via* breast milk has been well established in case of MMTV, HTLV-1 is transmitted probably by infected cells, not by free virions. I had little knowledge of the concentration of infected cells in breast milk. Furthermore, it was not easy to postulate the survival of infected T-cells in acidic conditions of the stomach.

This prompted us to test the possibility of the presence of milk-borne transmission of HTLV-1.

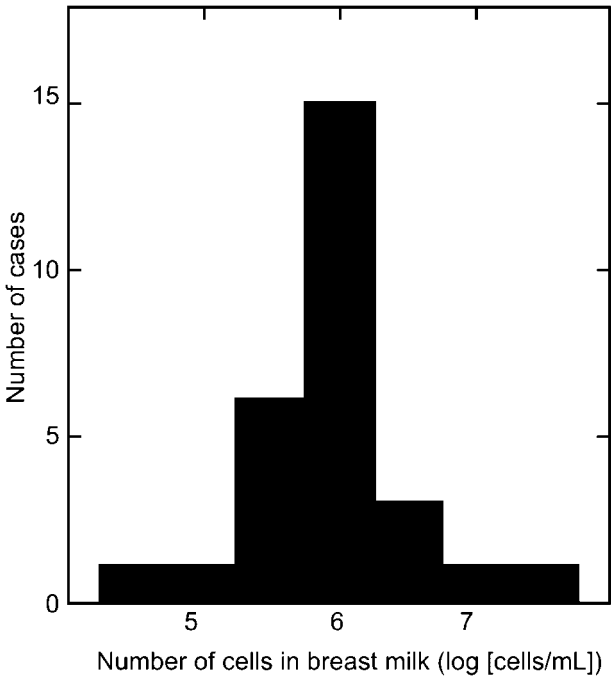

Fig. 1. Histogram of cell concentrations in breast milk from carrier mothers. Morphologically, 90% of them were macrophages. Roughly 1% of Ficoll/Conray-purified mononuclear cells were producing HTLV-1 antigens after culture for 4 weeks.

Doctors in the Department of Hematology joined to the study. Breast milk samples of carrier mothers were obtained. Surprisingly, the average concentration of cells in breast milk was as high as 10^6 cells/mL (Fig. 1), and approximately 90% of them were macrophages. After culture of mononuclear cells, purified by the Ficoll/Conray method, 0% to 10% of cells were found HTLV-1 antigen-positive, *i.e.*, infected with HTLV-1. Thus, the average number of HTLV-1 infected cells in every milliliter of breast milk derived from carrier mothers was computed to reach roughly 10^3. Because a baby can take a liter of breast milk a day, and will be breast-fed at least for 100 days, the total amount of breast milk a baby ingests will be over 100 liters, and the number of infected cells a weanling baby can receive will exceed 10^8.[19)]

Once the presence of cells infected with HTLV-1 in the breast milk of carrier mothers was confirmed with a dose quantitatively enough for establishing the infection, the next step was to obtain a hard evidence that the oral intake of infected cells can lead to infection. Most easily conceivable locations for homing of infected maternal T-cells in the baby are oropharyngeal lymphatic tissues or intestinal Peyer's patches. One of the major questions was if infected cells in breast milk can survive through the acidic condition in stomach of babies. Although some colleagues stated that stomach of babies should be less acidic than in adults, we knew that milk thrown up by babies is usually coagulated, suggesting the acidic condition of stomach even in newborn babies. Lymphocytes within the coagulated milk may survive even in the acidic condition. If the target location is oropharyngeal lymphatic tissues, the acidic barrier in the stomach is irrelevant. But I could not work out an experimental system to disclose the port of entry.

Because experiments in human babies are out of question, we started to seek out animal species for experimental infections. Preliminary experiments with mice and rats were not hopeful. A veterinarian friend taught me that handling of guinea pig babies is extremely difficult. Transfusion experiments of HTLV-1 in rabbits had been reported by Prof. I. Miyoshi,[20)] but we wanted to try experiments in primate species, closer to human species. However, presence of antibody against simian T-lymphotropic virus (STLV) was reported in some of Old World monkeys, which might interfere with the diagnosis of experimental infection.[21)] The age of separation of Africa and South America was estimated at 115–125 m.y,[22)] much older than that of separation of human and African apes at 4–5 m.y.[23)] We decided to use common marmosets, commercially available New World monkeys, hoping that they had not been contaminated with STLV and that they are susceptible to HTLV-1. Because preparation and handling of baby marmosets were extremely difficult, adult marmosets were used in experiments. Our boss, Prof. T. Miyamoto, allowed us to invest a fairly large size of money to buy marmosets and caging systems to start the infection experiment in primates, before outside research funds became available. We were lucky that the construction of a brand new building for animal experiments was just finished in the Medical School. After arrival of the marmosets, I was relieved to find that none of four marmosets we bought had the antibody reacting with HTLV-1.

Marmosets were fed with concentrated cell suspension to mimic breast-feeding using a 1-mL plastic syringe without needle. Because it took at least four weeks for culture to estimate the number of infected cells in breast milk, it was impossible to control the infection dose by breast milk. We first inoculated MT-2 cells producing a large amount of HTLV-1 to control the inoculum size of infected cells. In total, 7×10^7 cells were given in several separate

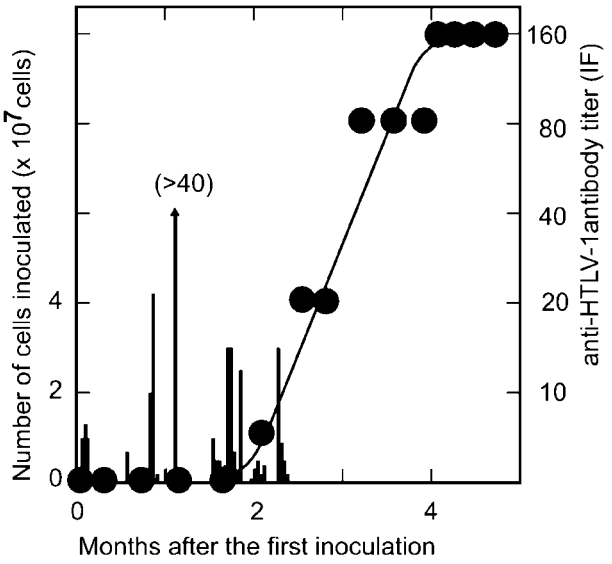

Fig. 2. Concentrated fresh human-milk cells donated by carrier mothers were inoculated orally in separate doses to common marmosets by plastic syringes without needle. Abscissa: months after first inoculation, Ordinate: total numbers of cells inoculated (bars); antibody titers against HTLV-1 antigen determined by the indirect immunofluorescence test (dots).

doses. At 2.5 months after the first inoculation, one of two marmosets started to show serum antibody activities against HTLV-1 by the indirect immunofluorescence test and by the immunoprecipitation test using ^{35}S-labled HTLV-1 antigens. The culture of peripheral blood was positive for infected cells, and the marmoset remained positive for years. The other marmoset remained negative both in culture and for antibodies. This proved that the oral intake of infected cells can establish HTLV-1 infection with the dose of $<1 \times 10^8$ infected cells in common marmoset.[19)]

In the second step, two more marmosets were inoculated with concentrated cell suspension from fresh breast milk donated by carrier mothers (Fig. 2). Again one of the two marmosets gained the seroconversion and positive cultures at 2.5 months after the first inoculation. This confirmed the working hypothesis that HTLV-1 can be transmitted by oral intake of breast milk from carrier mothers to their babies.[24)] This was supported by a retrospective analysis of babies born to carrier mothers in whom none of 10 completely bottle-fed babies had been infected.[25)] Recently, Hisada *et al.* reported that the higher concentration of the HTLV-1 infected cells in breast milk is significantly associated with the higher risk of milk-borne transmission of HTLV-1.[26)] By the way, because these infected marmosets were males, milk-borne transmissions of HTLV-1 in marmosets could not be examined.

Preparations for the intervention study

The last and final stage to study the significance of MTCT in the HTLV-1 endemy was an intervention, by stopping breast-feeding by carrier mothers and to look for the lack of transmission. Our hypothesis will be proven if the refraining from breast-feeding in carrier mothers significantly reduces the incidence of MTCT. Theoretically, I thought this intervention study not difficult, because we were not administering any additional chemicals to babies except for the requirement of formula-feeding. However, we encountered several serious difficulties to start the actual intervention study.

The most important hurdle was the nationwide project to recommend breast-feeding. The program was strongly supported by obstetricians, pediatricians and psychologists, and by the government. Medically, breast-feeding has been described of its importance by several reasons. The IgA-antibody in breast milk is the major source of maternal immunological protection of babies from respiratory and intestinal infections. Bottle-feeding has been inferred as a risky practice in developing countries, because as many as a third of babies can be lost after malnutrition and infectious diarrhea. These factors are not critical in contemporary Japan. A large number of children have been raised with bottle-feeding without serious drawbacks. If a baby has serious immunological or other problems, responsible underlying conditions might be more important than the HTLV-1 infection. Another concept is that good mother-to-child relation is only formed by breast-feeding. They say that the good relations between mother and children were lost in the history when most mothers in Western countries started to choose bottle-feeding due to cosmetic reasons in 1960s. The first response I received at the local government, when I visited to explain our plans, was that "the local government can not endorse our plan because the plan is against the national project". He added that "he could help us if the Department of Health, the national government, authorized our plan."

Another aspect was that it seemed almost impossible to keep the confidentiality of maternal carrier status from their family members and neighbors, if they would not breast-feed the baby. Some carrier mothers wanted to keep the information secret from their mothers-in-law. Later, it was found

that a significant proportion of carrier mothers, especially those residing in rural areas, chose breast-feeding because of confidentiality from family members and local community. Some obstetricians and pediatricians instructed mothers not to bottle-feed, because breast-feeding is important than anything else and the incidence of ATL among carriers is rare. They believed a newspaper describing the predictive incidence of ATL at "**∼0.1%**". However, the real incidence is "**∼0.1% per year**", which computes "**∼5% in life**".

Severity of health problems incurred by ATL was not widely understood by doctors in those days even in Nagasaki. Most doctors practicing in obstetrics or pediatrics had never seen ATL patients. Some nurses knew ATL better than doctors in private clinics, because the group of nurses in the clinic exchanged health information of their elder family members: some of them lost family members from ATL.

Our small pilot study induced some confusion in obstetricians and mothers in the Prefecture, because mothers started to realize differences in information depending on obstetricians they visited. It took almost a year and a half to persuade doctors within the University and in practice at Nagasaki, as well as the local government, to start on a prefecture-wide intervention study. One of the important leaders outside of the university who organized this project was Dr. S. Miyauchi, the former Nagasaki Prefecture Branch Secretary of Japan Association for Maternal Welfare. Some doctors and people in Nagasaki were really enthusiastic to start the first in the world experiment, named ATL Prevention Program (APP) Nagasaki, because they wanted to keep the ambition that Nagasaki has cherished to become the leader in medical science of Japan. Nagasaki University started as the first medical school of Western medicine in Japan, since Nagasaki was the only port in Japan open to Western cultures for ∼200 y until 1850s. Before actually starting the project, we gave a series of lectures introducing the system of the APP Nagasaki to obstetricians and health practitioners in the Nagasaki Prefecture.

The APP Nagasaki was commenced in July of 1987 in the whole Nagasaki Prefecture harboring a population of 1.5 million. Pregnant women were screened for anti-HTLV-1 antibody during the last trimester for the anti-HTLV-1 antibody. The last trimester was selected to minimize artificial abortions because of realizing the carrier status of HTLV-1. Fuji Rebio's particle agglutination assay was used for screening at commercial laboratories. All the positive and undetermined samples were collected and sent to our department to standardize the prefecture-wide diagnosis. This was found important, since some commercial laboratories reported false-positive results more often than others. The confirmation test consisted of titration of the anti-HTLV-1 antibody with the particle agglutination method and an indirect immunofluorescence test. Later, the western blot assay was added for the confirmation.

Pregnant women with positive results confirmed were informed as HTLV-1 positivity and recommended to refrain from breast-feeding. Children born to carrier mothers were asked to come back for blood test at 6, 12, 24 and 36 months after the delivery to examine possible infection. The purposes of the follow-up study were to estimate the MTCT incidence of HTLV-1, and to find out an appropriate time point after the delivery for the diagnosis of MTCT. Mothers were able to select breast-feeding, if they wanted to do so. If they chose it, we informed them that infected cells in breast milk can be inactivated by heat treatment at 60 °C for 30 min, or by freezing overnight. However, we strongly recommended the total abolishment of breast-feeding, because treatment of suctioned milk should be continued until weaning and the practice will not merit direct suckling with the baby during breast-feeding. Most carrier mothers who selected bottle-feeding received bromocriptine to minimize the unpleasant experience with breast milk production.

Prevalence of HTLV-1 carriers among pregnant women in Nagasaki

When we started the screening in the early 1980s, the prevalence of HTLV-1 carriers among pregnant women was approximately 5%, it declined to 1% level in the last decade (Fig. 3).[27] The total number of deliveries in the Nagasaki Prefecture also declined recently. The number of declined from ∼15,000 in 1987 to ∼12,000 in the last decade. Approximately three quarters of pregnant women were enrolled to the APP Nagasaki during this period. The decrease was consistent with the general tendency of fewer children in each family. The number of carriers detected among pregnant women dropped rather dramatically, from ∼1,000 to ∼100 in the same period.

This effect was reflected in the decline of prevalence dependent on the date of birth cohort in pregnant women (Fig. 4). Among pregnant women screened and born in the late 1940s through 1980, the

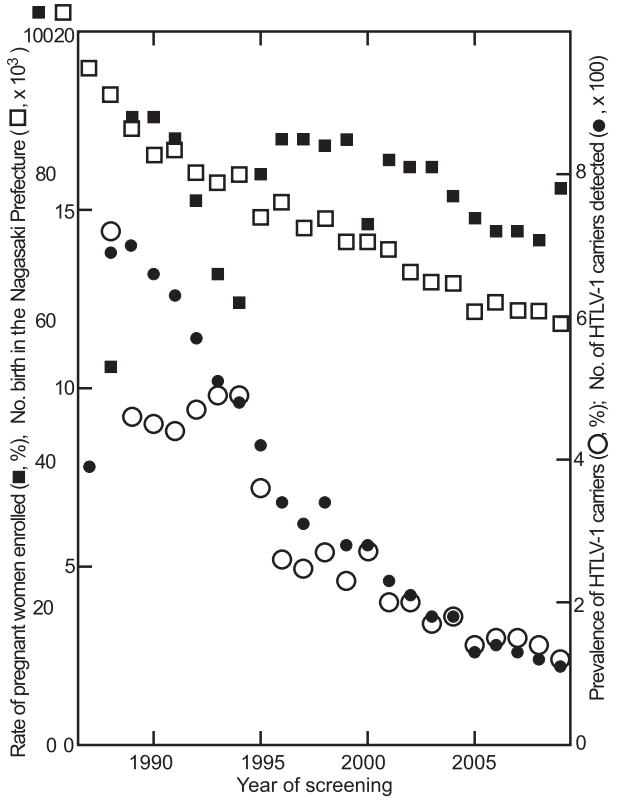

Fig. 3. Screening of pregnant women for the anti-HTLV-1 antibody by the ATL Prevention Program Nagasaki commenced in 1987. Numbers of deliveries in the Nagasaki Prefecture, pregnant women screened, carriers detected and the prevalence of carrier women in each year are shown. Data were taken from the reference[26] with permission of the Prefecture and authors.

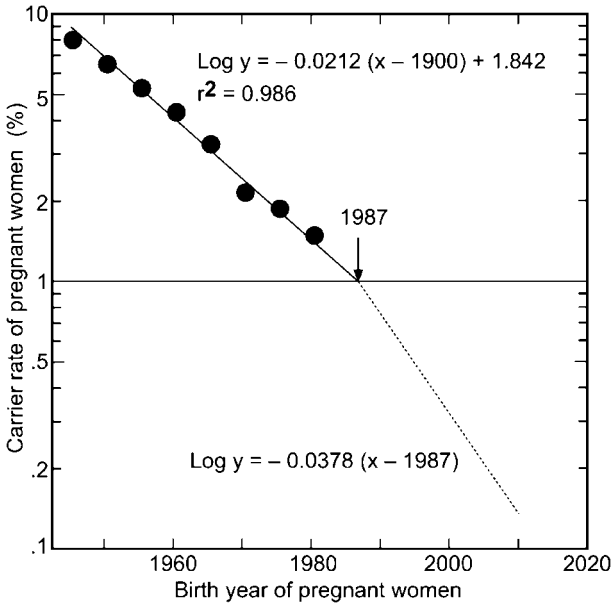

Fig. 4. Birth-cohort effect on the prevalence of HTLV-1 carriers among pregnant women in the Nagasaki Prefecture. The dotted line estimates sharper decline of the prevalence after the start of APP Nagasaki.

prevalence declined sharply with birth year. By the slope of regression line and the period of one generation computed by the average age of pregnant women at 29 y, the transmission rate from one generation to another is calculated to be 24%, which was well consistent with the average transmission rate by breast-feeding at ∼20% (Fig. 4). The prevalence in those born in 1987 was estimated at 1% from the graph. After the commencement of the APP Nagasaki in 1987, the decline in prevalence by cohort effect will be estimated to proceed even sharper.

Reason for the decrease is not clear, and may be associated with a number of factors. One of likely reasons is westernization of the community. Duration of breast-feeding period may become shorter in these days, because women at work tend to finish breast-feeding early and replace for bottle-feeding. The hygienic status has been dramatically improved to push up the immunity against infectious agents.

Table 3. Effects of feeding practice on mother-to-child infection of HTLV-1 in children born to carrier mothers in terms of bottle feeding, and breast-feeding for <6 months or ≥6 months[a]

Feeding method	Tested	Infected		P
	No. cases	No. cases	Incidence (%)	Chi squared test
Bottle-feeding	1,152	29	2.5	—[b]	
Breast-feeding					
<6 months	202	15	7.4	<0.001	—[b]
≥6 months	365	74	20.3	<0.001	<0.001

a) Prospectively followed cases only, born in the period from 1987 to 2004. Data were taken from the reference[27] with permission of the Prefecture and authors.
b) Base category being compared with.

Because a quarter of uninfected wives married to infected husband will acquire HTLV-1 within several years, larger family size in the past might have been a factor for increased prevalence in children.

Breaking the pathway of breast-feeding

Even after commencing the APP Nagasaski, the incidence of MTCT with HTLV-1 continued to remain at the level of 20% among mothers who chose

breast-feeding.[28),29)] Some gynecologists/pediatricians still strongly recommended breast-feeding than anything else. Some of mothers might be afraid of revealing their carrier status to their family members and/or neighbors, by refraining of breast-feeding. By shutting off the breast-feeding by carrier mothers, the MTCT dropped to the level of 2.5%, which was significantly lower than that in the group breast-fed ≥6 months ($P \leq 0.001$) (Table 3). The results were consistent with a working hypothesis that breast milk is the major route of the MTCT with HTLV-1 in the endemic area.

Reduction of HTLV-1 infection by short-term breast-feeding

In the meantime, other groups reported that early withdrawal of breast-feeding will decrease the incidence of MTCT significantly.[26),30),31)] Takahashi *et al.*[30)] described that the transmission rates of short-term (≤6 months) and long-term (>6 months) breast-feeders were 4.4% (4/90) vs. 14.4% (20/139), $P = 0.018$. They further wrote that the seroconversion rate of short-term breast-fed children was nearly equal to that of bottle-fed children; (4.4% [4/90] vs. 5.7% [9/158], $P = 0.471$). Instead, they should have described the lack of significant differences between short-term breast-fed and bottle-fed children, probably due to the small sample size. The statement they posted implied that short-term breast-feeding is as safe as bottle-feeding without any scientific evidence. Furthermore, most of children breast-fed for short-term or long-term were enrolled retrospectively, and we know that informations of breast-feeding period for previously born children are not very reliable. A newspaper dared to report no differences in the incidence of HTLV-1 infection between short-term breast-feeders and bottle-feeders, and many doctors even in the Nagasaki area believed the news.

Hirata *et al.* reported that bottle-fed children, breast-fed ≤3 months and >3 months had the incidence of infection in 12.8% (10/78), 5.1% (2/37) and 27.6% (16/42): an incidence in bottle-fed children higher than that in short-term breast-fed children was hard to explain except for statistical insignificance due to the small sample size.[31)] They implicated the association of anti-p40^{tax} antibody with the high risk of vertical transmission of HTLV-1; however, the notion was denied in another report.[26)] Another group reported similar observation of the infection in 2.5% (4/162), 3.9% (2/51) and 20.3% (13/64) in children with bottle-feeding, breast feeding for ≤6 months and for >6 months, respectively.[32)] The infection in bottle-fed and short-term breast-fed children was significantly lower than that in long-term breast-fed children. However, the difference in incidence between bottle-fed and short-term breast-fed children was insignificant, because of the small sample size of studied children. The breast-fed children for the short-term resulted in the incidence of MTCT lower than the breast-fed children for the long-term ($P = 0.021$, by the chi-squared test with the Yates' correction).[33)] However, some breast-fed children for the short-term seroconverted after the paper was submitted in a personal communication from one of the authors. Nevertheless, none of these reports were confirmed with the extension of follow-up data since then.

The APP Nagasaki kept the position to recommend bottle-feeding than short-term breast-feeding, because accumulating lines of evidence kept suggesting that the former is safer than the latter, although the data did not reach the statistical significance due to a small sample size. In 2008, the Nagasaki Prefecture and the APP Nagasaki published a recent report as "Report of joint study on the prevention of ATL by blocking mother-to-child transmission of HTLV-1: experiences and perspectives of 20 years".[27)] The data confirmed the incidence of HTLV-1 infection in the bottle-fed children was significantly lower than that in the breast-fed children for long-term (breast-fed for ≥6 months) (2.5% vs. 20.3%, $P < 0.001$) (Table 3). Among breast-fed children, the incidence was significantly lower in those breast-fed for short-term (<6 months) than that in those for the long-term (7.4% vs. 20.3%, $P < 0.001$), consistent with our previous reports.[28),29)] However, the significantly higher incidence in the short-term group than that in the bottle-fed group was clearly established in this recent report (7.4% vs. 2.5%, $P < 0.001$).[27)] The information spread through newspaper was found untrue, because the interpretation of previous papers was not scientific. Although children breast-fed for <6 months has significantly lower incidence of HTLV-1 infection than those breast-fed for ≥6 months, their chances of infection are significantly higher than those of bottle-fed children. Thus, the APP Nagasaki is keep recommending the exclusive bottle-feeding for carrier mothers.

Secondary pathway of MTCT

Even with complete bottle-feeding, 2.5% of babies born to carrier mothers were infected. Cord-blood samples of babies born to carrier mothers were tested for the HTLV-1 provirus by PCR. A short

Table 4. HTLV-1 infection markers, antibody and PCR, in cord-blood at birth and of peripheral blood at 6 or ≥12 months of age

ID of case	Cord blood		at 6 months		at ≥12 months	
	Ab	PCR	Ab	PCR	Ab	PCR
1	+	+	−	nt[a)]	−	−
2	+	+	−	nt	−	−
3	+	+	nt	nt	−	−
4	+	+	+	−	−	nt
5	+	+	+	−	nt	nt
6	+	−	−	nt	+	+
7	+	−	nt	nt	+	+
8	+	−	+	nt	+	nt
9	+	−	−	nt	+	nt

a) nt: not tested.

Table 5. Summery of APP Nagasaki: Screening and prospect

	Estimated	No. cases	Calculated
Total pregnancies in the Nagasaki Prefecture (1988–2006)		281,324	
Screened by APP Nagasaki		208,463	74%
Positives		7,265	3.5%
Bottle-fed		6,500	90%
Estimated infection, without intervention[a)]	24%		
with bottle-feeding[b)]	2.5%		
Estimated reduction by APP Nagasaki			
Infection[c)]	20%	5,200	
ATL incidence in future[d)]	5%	260	

a) Calculated from the decline of HTLV-1 carriers among the birth cohort of pregnant women (see Fig. 4). The value presented in Table 3 is 20.3% which is represented by much smaller number of samples.
b) See Table 3.
c) Rounded from the calculation of 24% − 2.5%, in a) and b).
d) The average life-time incidence of ATL among carriers: *ca.* 5%.[9),36)]

PCR to detect a part of the *pX* gene was positive in 4% of samples, and most positive signals were found to represent defective proviruses.[34),35)] Although in a small scale of cases, none of babies PCR-positive in the cord-blood kept the positive signal at 6 months after the delivery. On the other hand, none of babies who were diagnosed with infection beyond 12 months of age had been PCR-positive in their cord-blood (Table 4). These results suggest that intrauterine transmission of HTLV-1 should be rare, if any. The results were consistent with our early observations that none of cord-blood samples were positive for HTLV-1 with neither IgM-antibody nor HTLV-1 antigen in culture. I prefer transmission during the delivery to transplacental transmission, as is the case for other viruses, such as HBV and HCV.[12)]

Is it possible to eliminate HTLV-1 from the endemic area?

Even without interventions, sooner or later, HTLV-1 will be eliminated from the endemic area. A large number of Japanese were emigrated from endemic areas, for example to Hawaii, California, Canada and South America. However, HTLV-1 is not markedly endemic among Japanese descendants in those districts any longer. Even in Nagasaki, the prevalence among pregnant women was approximately 5% in the early 1980s, but declined to 1% in the last decade (Fig. 3). This decline is not related to our intervention, because they had been born before commencing the APP Nagasaki. When the prevalence in pregnant women was analyzed by birth cohorts, the carrier rate decreased linearly in the logarithmic scale during the era without intervention (Fig. 4). The slope indicated that the prevalence decreased to 24% in each generation when the generation time was computed as 29 years using the average age of pregnant women in the APP study. The rate of decline was consistent with the observed rate of MTCT with long-term breast-feedings at approximately 20%.

By the end of 2006, >200,000 screening tests were performed for the detection of HTLV-1 carriers which covered approximately 74% of total pregnancies in the Nagasaki Prefecture during the period, and 7,265 tests were positive (3.5%)[27)] (Table 5). Since approximately 90% of the positive mothers agreed to refrain from breast-feeding to minimize the chance of HTLV-1 transmission, approximately 6,500 children born to carrier mothers were bottle-fed in the intervention by the APP Nagasaki. The transmission rate was calculated to be 24% from the slope of reduction of carrier women calculated from birth cohort (Fig. 4), and the transmission rate of bottle-feeding was 2.5% (Table 4), approximately 20% of putative transmission was blocked by the APP Nagasaki. Therefore, it can be estimated that approximately 1,300 potential transmissions and 260 potential ATL cases in future were prevented in Nagasaki by the year 2006. Because the prevalence of pregnant carrier has been declining, the number of these protected cases will also decline in the future.

Nationwide screening for HTLV-1

New ATL cases have been developing among HTLV-1 carriers.[36] To minimize the further expansion of the carrier pool, introduction of effective measures has been awaited outside of the Nagasaki Prefecture. In October, 2010, the nationwide screening policy of pregnant women for HTLV-1 has been announced,[37] but details are unknown. In our experiences, there are several potential problems for the program. Education of obstetric doctors is essential. If attending doctors do not understand the severity of ATL, it is difficult to deliver the appropriate information for pregnant women and to persuade them diagnosed positive toward refraining from breast-feeding. Most obstetricians and pediatricians, especially those in non-endemic areas, have no experiences with ATL. Most of them may think ATL would be treatable in the current medical standards, just like the other leukemias, and the incidence of ATL among carriers would be rare. In our experiences, most pregnant women visiting certain doctors selected breast-feeding rather than bottle-feeding, probably because they recommend breast-feeding as the most important tool for rearing babies. On the other hand, if mothers in non-endemic areas do not breast-feed their baby, their carrier status may be revealed in the community just because of bottle-feeding. It must be reminded that efforts for community education were far from the satisfactory level, even for HIV. If carrier mothers do not refrain from breast-feeding, the program will only be a political excuse.

Consideration for cost performance of the screening

Costs of anti-HTLV-1 antibody detection for screening and confirmation are approximately 2,000 JYE and 5,000 JYE, respectively. Since the prevalence is usually less than 5%, the total cost is mainly dependent on the screening level. The incidence of HTLV-1 infection among breast-fed children and that of ATL among carriers are computed as approximately 20% and 5% during the life. When we started the APP Nagasaki in the late 1980s, the average prevalence was 4%. The computed costs for detecting a HTLV-1 carrier, for preventing an infection and for reducing a case of ATL development were 50,000, 250,000 and 5,000,000 JYE, respectively. In 2010, the average prevalence dropped to the 1% level even in Nagasaki, the screening cost increased by four-fold. In non-endemic areas with the prevalence of 0.1%, the screening cost will be 40-fold of that originally computed in Nagasaki 30 y ago, *i.e.*, 200,000,000 JYE to prevent a case of ATL that might develop one half century later.

Risk of false diagnosis

False positive and false negative diagnoses can happen. If the false positive diagnosis occurs in 0.1% of tested, and the real prevalence is 5%, the incidence of false diagnosis among real positives is limited to 2%. However, in the area with real prevalence of 0.1% (most areas outside of Kyushu have the prevalence below this level), as much as one half of pregnant women diagnosed to be positive can be false positive. Notice of HTLV-1 carrier status itself can induce a strong trauma, and positive mothers are forced to decide the feeding method. I do not know how the government has decided to carry out the confirmation test. It should be performed at accredited institutions. Because positive samples will be relatively rare, especially in non-endemic regions, confirmation tests performed at small local laboratories are not reliable, which may further increase the rate of false positives. It is likely that the government will pay for the screening, but who will pay for the confirmation test which is more expensive than screening? If the confirmation test will not be covered, screened positives may be hardly to be confirmed. I hope the program will be commenced without much confusion.

Acknowledgements

The author deeply thanks to a number of collaborators inside and outside of Nagasaki University. The study could not be initiated or conducted for such a long period without cooperation of participated pregnant women, obstetricians, pediatricians and health practitioners throughout the Nagasaki Prefecture. The Nagasaki Prefecture has kept supporting the intervention study of APP Nagasaki since 1987. Part of this study was supported by the Grant-in-Aid for Cancer Research by the Ministry of Education Science and Culture, Japan, for over 20 years.

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(Received Dec. 2, 2010; accepted Feb. 24, 2011)

Profile

Shigeo Hino was born in 1942. While he was a medical student at the Faculty of Medicine, University of Tokyo, he spent his after school hours regularly at the Department of Oncology, Institute of Infectious Diseases, University of Tokyo, headed by Prof. Tadashi Yamamoto. He became interested in cancer immunology. Following a one-year internship (the last year of having internship in Japanese System), he became a graduate school student from 1968 to 1972 at Prof. Yamamoto's laboratory where he studied retroviral oncogenesis. Upon graduation, he joined the Department of Viral Infection, Institute of Medical Science headed by Prof. Minoru Matsumoto. He was awarded the Fogarty International Fellowship by the National Institute of Health, Bethesda MD, and studied comparative immunochemistry of mammalian retroviral glycoproteins sponsored by Dr. Stuart A. Aaronson (Molecular Biology Section, Viral Carcinogenesis Bransch, National Cancer Institute) from 1974 to 1977. In 1980, he joined Department of Bacteriology, School of Medicine, at Nagasaki University headed by Prof. Tsutomu Miyamoto, where he started to work with the transmission pathway of HTLV-1. He worked as a professor at the Department of Virology, School of Medicine, Tottori University from 1991 to 2008. He was awarded the Kojima-Saburo Memorial Culture Award in 1992, the Princess Takamatsu Cancer Research Award in 2002, and the Nagayo-Matasaburo Award by the Japanese Cancer Association in 2006, for establishing breast-feeding by HTLV-1 carrier mothers as the major transmission pathway of HTLV-1 and primary prevention of ATL by refraining from breast-feeding. He was nominated as an honorary member of Japanese Cancer Association in 2007.